

# CD5 Expression Is Developmentally Regulated By T Cell Receptor (TCR) Signals and TCR Avidity

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## Summary

Recent data indicate that the cell surface glycoprotein CD5 functions as a negative regulator of T cell receptor (TCR)-mediated signaling. In this study, we examined the regulation of CD5 surface expression during normal thymocyte ontogeny and in mice with developmental and/or signal transduction defects. The results demonstrate that low level expression of CD5 on CD4<sup>-</sup>CD8<sup>-</sup> (double negative, DN) thymocytes is independent of TCR gene rearrangement; however, induction of CD5 surface expression on DN thymocytes requires engagement of the pre-TCR and is dependent upon the activity of p56<sup>lck</sup>. At the CD4<sup>+</sup>CD8<sup>+</sup> (double positive, DP) stage, intermediate CD5 levels are maintained by low affinity TCR–major histocompatibility complex (MHC) interactions, and CD5 surface expression is proportional to both the surface level and signaling capacity of the TCR. High-level expression of CD5 on DP and CD4<sup>+</sup> or CD8<sup>+</sup> (single positive, SP) thymocytes is induced by engagement of the  $\alpha/\beta$ -TCR by (positively or negatively) selecting ligands. Significantly, CD5 surface expression on mature SP thymocytes and T cells was found to directly parallel the avidity or signaling intensity of the positively selecting TCR–MHC–ligand interaction. Taken together, these observations suggest that the developmental regulation of CD5 in response to TCR signaling and TCR avidity represents a mechanism for fine tuning of the TCR signaling response.

Key words: CD5 • thymocyte • development • signal transduction

CD5 is a monomeric cell surface glycoprotein expressed on thymocytes, all mature T cells, and a subset of B cells, B-1 cells (1–4). Putative CD5 ligands include CD72, a pan-B cell antigen, and CD5L, a recently described protein expressed on activated splenocytes, B cells, and activated murine T cell clones (5, 6), suggesting that CD5 may be involved in regulating immune cell interactions. The cytoplasmic domain of CD5 contains three potential tyrosine phosphorylation sites, including a putative ITAM (immunoreceptor tyrosine-based activation motif)<sup>1</sup> or ITIM (immunoreceptor tyrosine-based inhibition motif) sequence (4, 7) and multiple potential Ser/Thr phosphorylation sites (4). After TCR engagement, CD5 is tyrosine phosphorylated and becomes associated with a multimolecular complex that may include TCR- $\zeta$ , CD2, CD4, CD8, p56<sup>lck</sup>, p59<sup>lyn</sup>, PTPC1, and Zap70 (8–12). The physiologi-

cal role of CD5 is still not clearly understood. Previous studies have shown that treatment of T cells with anti-CD5 enhances TCR-mediated activation, proliferation, and IL-2 production (13–15). On the other hand, more recent data indicate that CD5 acts to negatively regulate signaling through both the B and T cell antigen receptors (16, 17). In the absence of CD5, peritoneal B-1 cells, which normally are triggered to undergo apoptosis in response to mIgM cross-linking, develop resistance to apoptosis and enter the cell cycle (17). Likewise, thymocytes from CD5<sup>-/-</sup> mice are hyperresponsive to stimulation through the TCR, and the efficiency of thymocyte selection in CD5<sup>-/-</sup>,  $\alpha/\beta$ -TCR transgenic mice is altered in a manner consistent with enhanced TCR signaling (16).

CD5 surface expression is tightly regulated throughout T cell development. Low levels of CD5 are expressed on immature CD4<sup>-</sup>CD8<sup>-</sup> (double negative, DN) thymocytes. CD5 surface expression then increases at both the CD4<sup>+</sup>CD8<sup>+</sup> (double positive, DP) and CD4<sup>+</sup> or CD8<sup>+</sup> (single positive, SP) stages and relatively high levels of CD5 are maintained on circulating SP T cells (3, 18). In this

<sup>1</sup>Abbreviations used in this paper: DN, CD4<sup>-</sup>CD8<sup>-</sup> double negative; DP, CD4<sup>+</sup>CD8<sup>+</sup> double positive; SP, CD4<sup>+</sup> or CD8<sup>+</sup> single positive; FCM, multicolor flow cytometry; ITAM, immunoreceptor tyrosine-based activation motif.

study, we sought to identify the cellular mechanisms regulating CD5 expression during development. Our results demonstrate that CD5 is upregulated at crucial points during thymocyte development by pre-TCR and TCR engagement and that the level of CD5 surface expression is directly related to pre-TCR and TCR signaling intensity. Significantly, CD5 surface levels were found to vary considerably among mature SP thymocytes and T cells that express distinct TCRs, and the level of CD5 expression paralleled the avidity of the positively selecting TCR-MHC-ligand interaction. Together, these results suggest that the ability to regulate CD5 surface expression in response to TCR signaling is important for fine tuning the TCR signaling response and for selection of the mature TCR repertoire.

## Materials and Methods

### Mice

C57 BL/6 (B6) mice were bred within our facility. Mutant strains of mice used for this study included Rag2<sup>-/-</sup> (19); MHC class I<sup>-/-</sup> (β2M<sup>-/-</sup>; reference 20); MHC class II<sup>-/-</sup> (Aβ<sup>-/-</sup>; reference 21); MHC class I × II<sup>-/-</sup> (β2M<sup>-/-</sup> × Aβ<sup>-/-</sup>; reference 22); TCR-α<sup>-/-</sup> (23); and lck<sup>-/-</sup> (24). α/β-TCR transgenic lines included P14 (25), H-Y (26), AND (27), and DO11.10 (DO10; 28). TCR-ζ chain transgenic and TCR-ζ<sup>-/-</sup> mice were generated as previously described (29, 30). For positive selection experiments, mice were bred to C57 BL/6, B10.D2 or B10.A(5R) mating partners to change the selecting haplotype.

### Antibodies

mAbs used for flow cytometric analysis were purchased from PharMingen (San Diego, CA) and included fluorochrome (FITC or PE) or biotin-conjugated anti-Thy1.2 (53-2.1), anti-B220 (RA3-6B2), anti-CD4 (H129.19), anti-TCR-β (H57-597), anti-CD8α (53-6.7), anti-CD3ε (145-2C11), anti-CD5 (53-7.3), anti-CD69 (H1.2F3), anti-CD25 (7D4), anti-CD44 (IM7), anti-Vα11 (RR8-1), and anti-Vα2 (B20.1). Unconjugated anti-CD16/CD32 (2.4G2), and rat IgG2a,κ (R35-95) were used to block nonspecific Fc receptor binding and as control antibody, respectively. The anti-H-Y clonotypic receptor mAb (T3.70) and anti-DO10 clonotypic receptor mAb (KJ126) were purified from cell culture supernatants and labeled with FITC in our laboratory. Streptavidin Red 670 (GIBCO BRL, Gaithersburg, MD) was used in conjunction with biotinylated antibodies for flow cytometry.

### Flow Cytometric Analysis

Thymi and lymph nodes were excised from mice and single cell suspensions were prepared. For multicolor flow cytometry (FCM), thymocytes or lymph node cells were first incubated with antibody to the Fc receptor (mAb 2.4G2) to prevent nonspecific binding of antibodies. Background staining was measured using fluorochrome-conjugated rat IgG2a,κ and designated as control peaks. For two- and three-color FCM, cells were incubated with FITC-conjugated, PE-conjugated, and biotinylated antibodies, followed by the addition of Red 670 streptavidin. FCM was performed on a FACScan<sup>®</sup> using standard Cell Quest software (Becton Dickinson, San Jose, CA). Data were collected on 10<sup>4</sup> viable cells as determined by forward and side light scatter. Cell numbers

among the various thymocyte populations were normalized by gating and collecting on DN, DP, or SP thymocytes. CD5 levels on thymocyte subpopulations were measured by staining with CD5, CD4, and CD8 antibodies, followed by a three-color FACS<sup>®</sup> analysis and gating according to the CD4/CD8 profile.

### Purification of DN Thymocyte Populations

Total thymocytes from B6 mice were stained with a mixture of biotinylated anti-CD3, -CD4, -CD8, and -B220 mAb antibodies, washed, and incubated with Streptavidin microbeads (Miltenyi Biotec, Auburn, CA). DN (CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>B220<sup>-</sup>) thymocytes were then purified by magnetic separation according to the manufacturer's protocol (Miltenyi Biotec). Purity of magnetically separated DN thymocytes was >95% as assessed by FCM.

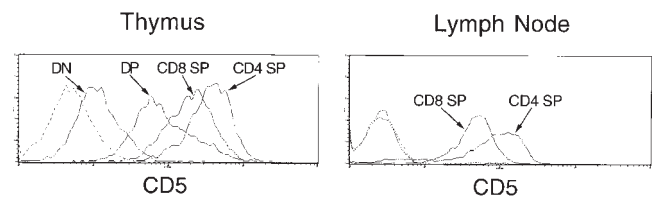
### Thymocyte Stimulation

**In Vitro CD3 Cross-linking.** 24-well plates were coated with 1–50 μg of anti-CD3ε (2C11) in PBS overnight at 4°C and subsequently washed with RPMI 1640 medium (Biofluids Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Labs. Inc., Logan, UT), 0.1 mM MEM nonessential amino acids, 1 mM MEM sodium pyruvate solution, 1% penicillin/streptavidin, and 5.5 × 10<sup>-5</sup> M 2-ME (GIBCO BRL). 4 × 10<sup>6</sup> thymocytes were resuspended in 2 ml of the supplemented RPMI medium and plated in the presence or absence of 2C11-coated wells for 18 h at 37°C 5% CO<sub>2</sub>. After cross-linking, thymocytes were spun, washed, and resuspended in FACS buffer (1 × HBSS, 1% BSA, and 0.1% NaN<sub>3</sub>) before staining with antibodies. When required, thymocyte viability was measured by propidium iodide staining, and data were collected on propidium iodide-negative cells.

**In Vivo CD3 Cross-linking.** Rag-2<sup>-/-</sup> mice were injected intraperitoneally with 1–10 μg 2C11 antibody/gram of body wt, or an equal volume of PBS alone as a control. Mice were killed 1, 3, or 9 d after injection and thymocytes were harvested and stained as described above.

## Results

**CD5 Surface Expression Is Upregulated on DN Thymocytes by pre-TCR Engagement.** Examination of thymocyte subsets from B6 mice reveals a stepwise progression in CD5 surface expression that correlates with thymocyte maturity (Fig. 1). Low levels of CD5 are expressed on immature CD4<sup>-</sup>CD8<sup>-</sup> (double negative, DN) thymocytes (CD5<sup>lo</sup>),

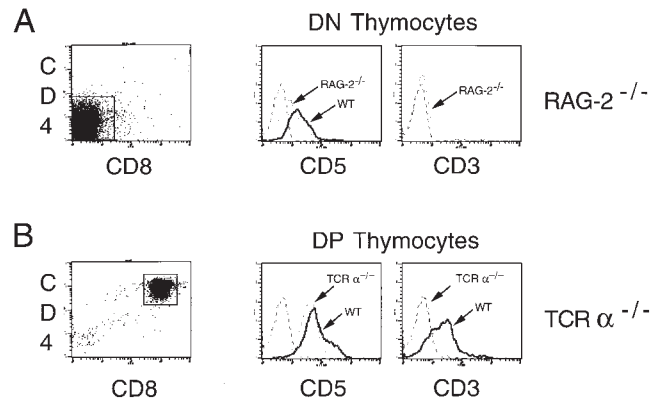


**Figure 1.** CD5 expression during normal T cell development. (A) Thymus. (B) Lymph node. Thymocytes and T cells from a C57 BL/6 mouse were stained with CD5, CD4, and CD8, and CD5 was assessed by gating on the various populations. Histograms represent CD5 expression on the indicated subpopulations. Dotted lines represent staining with control (nonspecific) antibody.

followed by an approximately sixfold increase in CD5 expression at the CD4<sup>+</sup>CD8<sup>+</sup> (double positive, DP) stage (CD5<sup>int</sup>), and a further three- to fivefold increase in CD5 expression as thymocytes reach the mature CD4<sup>+</sup> or CD8<sup>+</sup> (single positive, SP) stage (CD5<sup>hi</sup>). Relatively high levels of CD5 are maintained on mature peripheral SP T cells (Fig. 1), with the highest expression on CD4<sup>+</sup> T cells (Fig. 1). To investigate how CD5 expression is controlled during development, we began by examining thymocytes from mutant strains of mice that exhibit defects in T cell development, signal transduction, or both.

Thymocytes from mice deficient in expression of the Rag-2 gene product (Rag-2<sup>-/-</sup>) fail to develop beyond the DN stage and fail to express either pre-TCR or α/β-TCR surface complexes owing to a defect in the initiation of V-(D)-J recombination (19, 31). Yet thymocytes from Rag-2<sup>-/-</sup> mice express low levels of CD5 (Fig. 2). CD5 expression was also observed on TCR<sup>-</sup> DN thymocytes from TCR-β<sup>-/-</sup> mice (results not shown) demonstrating that neither the pre-TCR nor the α/β-TCR is required for low-level expression of CD5. DN thymocytes from mice in which TCR-α rearrangement is inhibited (TCR-α<sup>-/-</sup>) mature to the DP stage, but are unable to progress further in development (23). Significantly, DP thymocytes from TCR-α<sup>-/-</sup> mice express higher levels of CD5 than do DN thymocytes (Fig. 2). In fact, CD5 levels on the majority of DP thymocytes from control (TCR-α<sup>+/+</sup>) and TCR-α<sup>-/-</sup> mice are similar, differing mainly in the absence of a small CD5<sup>hi</sup> DP population in TCR-α<sup>-/-</sup> mice (Fig. 2; reference 32).

Since in the absence of TCR-α chain, thymocytes can express pre-TCR but not α/β-TCR complexes, we next investigated whether pre-TCR signals could upregulate CD5 surface expression. Expression of the pre-TCR begins at the DN CD44<sup>lo/-</sup> CD25<sup>+</sup> stage of development and is required for transition to the DN CD44<sup>-</sup>CD25<sup>-</sup> stage (33, 34). Examination of DN CD44<sup>-</sup>CD25<sup>-</sup> thymocytes revealed that these cells express three- to fourfold higher levels of surface CD5 than do their immediate precursors, DN CD44<sup>lo/-</sup> CD25<sup>+</sup> thymocytes (Fig. 3 A). Signals transduced by the pre-TCR induce cell cycle progression, resulting in the expansion of cells that have undergone productive β-rearrangement, a process termed “β selection” (34). Consequently, in normal mice, the DN CD44<sup>lo/-</sup> CD25<sup>+</sup> thymocyte compartment consists of two subsets (“E” and “L”) that can be distinguished on the basis of cell size (34). The E subset contains small resting cells that either have not yet undergone β selection or have failed to productively rearrange their β chain genes, whereas the L subset consists of larger cells that have received pre-TCR signals and have entered the cell cycle (reference 34; Fig. 3 A). Examination of CD5 surface expression on DN CD44<sup>lo/-</sup> CD25<sup>+</sup> thymocyte subsets revealed that L cells express approximately fourfold higher levels of CD5 than do E cells (Fig. 3 A). Collectively, these results indicate that signaling through the pre-TCR upregulates CD5 surface expression on DN thymocytes. To test this hypothesis further, we next determined the effect of CD3 cross-linking on CD5 expression. Injection of anti-CD3 antibodies into

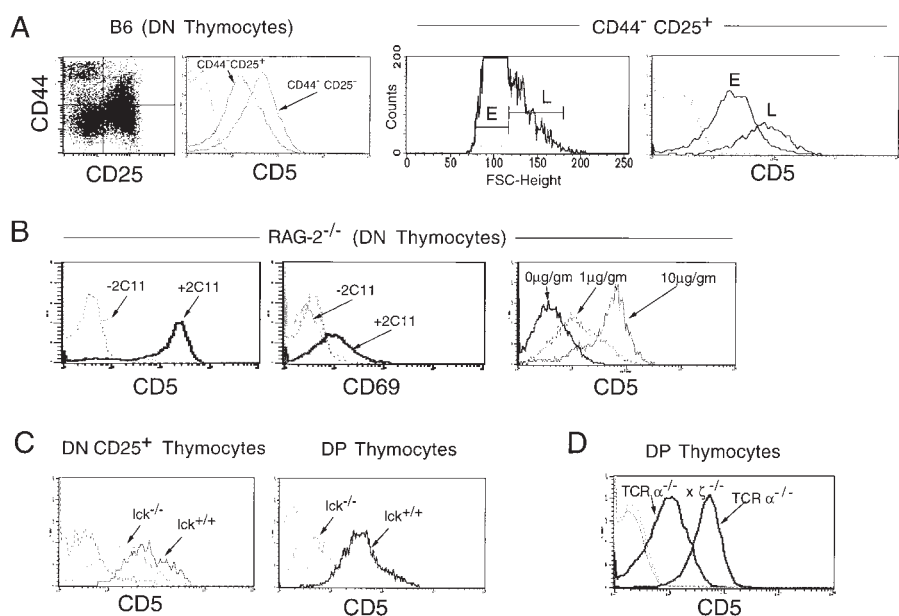


**Figure 2.** CD5 surface expression on thymocytes from Rag-2<sup>-/-</sup> and TCR-α<sup>-/-</sup> mice. Dot plots illustrate the CD4/CD8 staining profile and demonstrate the stage at which thymocyte development is blocked. Histograms depict CD5 and CD3 surface levels on gated CD4<sup>-</sup>CD8<sup>-</sup> (DN) thymocytes (A), or CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocytes (B). CD5 and CD3 surface expression on DN and DP thymocytes from control (WT) animals are included for comparison. Dotted lines represent control peaks.

Rag-2<sup>-/-</sup> mice has been shown to mimic pre-TCR signaling as it promotes the formation of DP thymocytes (35, 36). This response has been attributed to the low level expression of CD3 signaling subunits on the surface of Rag-2<sup>-/-</sup> thymocytes in the absence of β and pre-Tα chains (37). To ascertain whether direct engagement of these surface complexes could induce CD5 expression on DN thymocytes before their transition to the DP stage, Rag-2<sup>-/-</sup> mice were analyzed at multiple time points after intraperitoneal injection of anti-CD3 antibody. Significantly, as early as 24 h after anti-CD3 injection, thymocyte cellularity was increased fourfold and Rag-2<sup>-/-</sup> thymocytes, which still consisted entirely of DN cells, expressed high levels of CD5 (Fig. 3 B). Induction of the early activation antigen, CD69, was also observed on DN thymocytes 24 h after injection (Fig. 3 B). At 3 d after injection, CD5 levels on DN thymocytes remained high and large numbers of CD5<sup>int-hi</sup> DP thymocytes were present in the thymus of Rag-2<sup>-/-</sup> mice (data not shown).

Because signaling by the pre-TCR has been shown to be dependent upon the activity of the protein tyrosine kinase p56<sup>lck</sup> (24, 38), we also examined the importance of Lck for the induction of CD5 expression. DN, CD44<sup>lo/-</sup>CD25<sup>+</sup> thymocytes from lck<sup>-/-</sup> mice were found to express lower levels of CD5 than DN, CD44<sup>lo/-</sup>CD25<sup>+</sup> thymocytes from lck<sup>+/+</sup> mice (Fig. 3 C). In addition, although lck<sup>-/-</sup> mice contained α/β-TCR<sup>+</sup> DP thymocytes, these cells also expressed low levels of CD5 relative to DP thymocytes from lck<sup>+/+</sup> mice (Fig. 3 C).

To determine if induction of CD5 on DN thymocytes correlates with pre-TCR signal intensity, Rag-2<sup>-/-</sup> mice were injected with varying amounts of stimulating (anti-CD3ε) antibody. The results demonstrated a direct relationship between anti-CD3ε dosage and CD5 surface expression (Fig. 3 B). The relationship between pre-TCR signaling capacity and CD5 expression was also examined

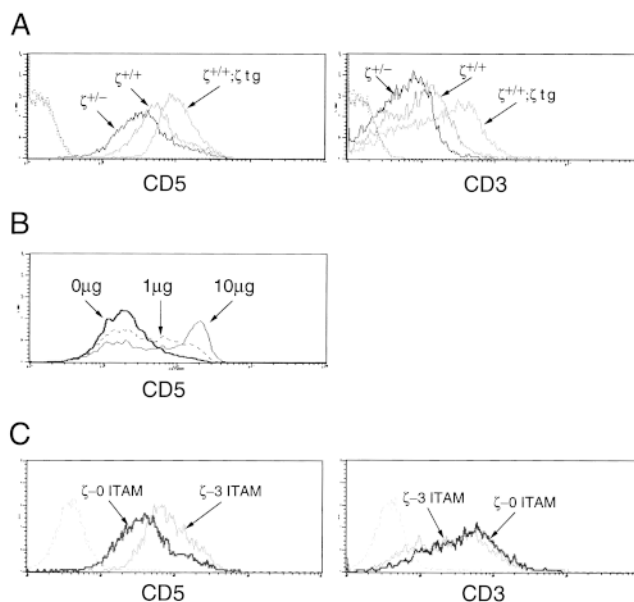


**Figure 3.** (A) CD5 surface expression on CD4<sup>-</sup>CD8<sup>-</sup> (DN) thymocyte subsets. DN thymocytes from B6 mice were isolated as described in Materials and Methods, and stained with CD25 and CD44 antibodies to distinguish the various DN subpopulations (dot plot). Histogram on the left shows CD5 expression levels on gated CD44<sup>-</sup>CD25<sup>+</sup> and CD44<sup>-</sup>CD25<sup>-</sup> thymocyte subpopulations. The middle histogram shows forward scatter profile of DN CD44<sup>-</sup>CD25<sup>+</sup> thymocytes from B6 mice (white histogram) or from Rag-2<sup>-/-</sup> mice (gray histogram). Rag-2<sup>-/-</sup> thymocytes were used for placement of E and L gates as shown. Right histogram shows CD5 expression on E and L gated DN CD44<sup>-</sup>CD25<sup>+</sup> thymocytes from B6 mice. (B) Induction of CD5 and CD69 surface expression on Rag-2<sup>-/-</sup> DN thymocytes by anti-CD3ε antibody. Left and middle: 200 µg of anti-CD3ε (2C11) antibody was injected into Rag-2<sup>-/-</sup> mice (+2C11) and CD5 (left) or CD69 (middle) surface levels on DN thymocytes were determined 24 h later. Control animals were injected with PBS (-2C11). Right: Rag-2<sup>-/-</sup>

mice were injected with 0, 1, or 10 µg 2C11/gram body wt and DN thymocytes were analyzed for CD5 surface expression 3 d later. (C) CD5 surface expression on DN CD25<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocytes from lck<sup>-/-</sup> mice. CD5 levels on DN CD25<sup>+</sup> and DP thymocytes from control (lck<sup>+/+</sup>) mice are included for comparison. (D) CD5 surface expression on gated CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocytes from TCR-α<sup>-/-</sup> and TCR-α<sup>-/-</sup> × ζ<sup>-/-</sup> mice. Dotted lines represent staining with control antibody.

by analyzing CD5 expression on DP thymocytes from TCR-α<sup>-/-</sup> × ζ<sup>-/-</sup> mice, which express pre-TCR in the absence of associated ζ chain. Previous work has shown that the pre-TCR is expressed and can function, though less efficiently, in the absence of ζ to promote the formation of DP thymocytes, indicating that ζ chain contributes quantitatively to the pre-TCR signaling response (29, 38). Indeed, DP thymocytes from TCR-α<sup>-/-</sup> × ζ<sup>-/-</sup> mice expressed significantly lower levels of CD5 than did DP thymocytes from TCR-α<sup>-/-</sup> mice (Fig. 3 D). Taken together, these results demonstrate that signals transduced by the pre-TCR induce the upregulation of CD5 on DN thymocytes, resulting in the formation of DP CD5<sup>int</sup> thymocytes.

**CD5 Expression on DP Thymocytes Is Maintained by α/β-TCR Engagement and Is Proportional to TCR Surface Levels and TCR Signaling Capacity.** CD5 surface expression has been shown previously to correlate with expression of the α/β-TCR during T cell development (3, 18). To investigate this relationship further, we used mice generated previously that express different levels of TCR due to variable expression of ζ chain (30). Examination of DP thymocytes from ζ<sup>+/-</sup> and ζ<sup>+/+</sup>; ζ tg mice, which express low or high levels of α/β-TCR relative to ζ<sup>+/+</sup> mice, respectively, revealed a direct relationship between TCR surface expression and mean CD5 surface expression on DP thymocytes (Fig. 4 A). To determine if TCR engagement was specifically required for CD5 expression on DP thymocytes, we then examined thymocytes from mice lacking MHC (class I × II)<sup>-/-</sup> in which development is arrested at the TCR<sup>+</sup> DP stage. Although most DP thymocytes from MHC class (I × II)<sup>-/-</sup> mice express higher levels of α/β-TCR than do DP thymocytes from control mice, these cells express low lev-

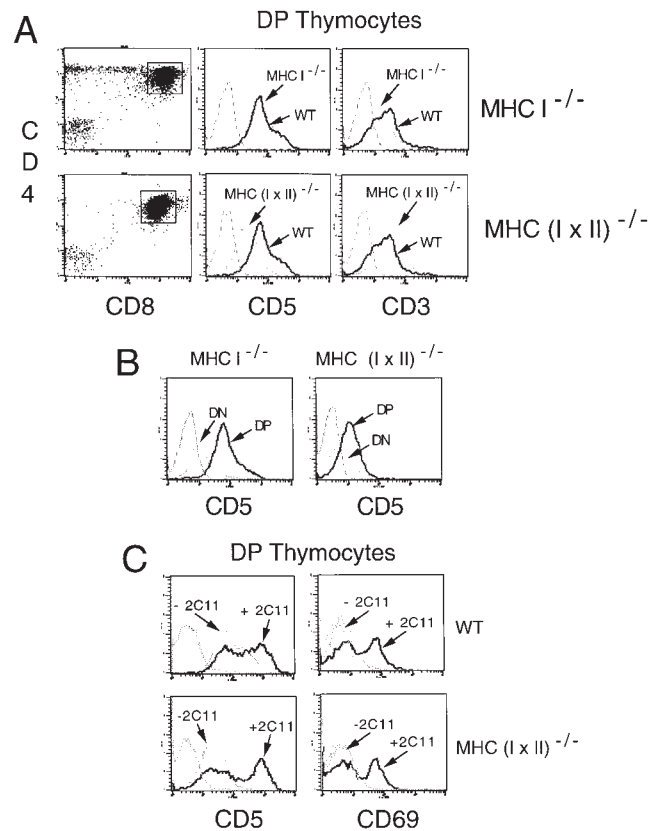


**Figure 4.** TCR levels and TCR signaling intensity influence CD5 expression on CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocytes. (A) Effect of TCR levels on CD5 expression. Histograms depict CD5 and CD3 expression on gated CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocytes from TCR-ζ heterozygotes (TCR-ζ<sup>+/-</sup>) and TCR-ζ-transgenic mice (TCR-ζ<sup>+/+</sup>; ζ tg) relative to control mice (TCR-ζ<sup>+/+</sup>). (B) Relationship between TCR signal intensity and CD5 expression. DP thymocytes were stimulated for 18 h on plates coated with the indicated amount of cross-linking antibody (anti-CD3ε; mAb 2C11) then analyzed for CD5 surface expression. (C) Effect of TCR signaling capacity on CD5 expression. Histograms depict CD5 and CD3 surface expression on DP thymocytes from TCR-ζ<sup>-/-</sup> mice reconstituted with transgenes encoding either full length ζ chain (ζ-3 ITAM) or ζ chain lacking sequences required for signal transduction (ζ-0 ITAM) (reference 30, see Results). Dotted lines represent staining with control antibody.

els of CD5 (Fig. 5 A). Indeed, the level of CD5 expressed on DP thymocytes from MHC class I  $\times$  II<sup>-/-</sup> mice is similar to that expressed on DN thymocytes (Fig. 5 B). In contrast, DP thymocytes from mice lacking expression of either MHC class I (Fig. 5, A and B) or MHC class II alone (data not shown) express normal (intermediate) levels of CD5. Thus, interaction of TCR<sup>+</sup> DP thymocytes with self-MHC appears to be required to sustain CD5 surface expression at levels above those observed on DN thymocytes. Consistent with this idea, anti-TCR cross-linking resulted in induction of both CD5 and CD69 on DP thymocytes from class I  $\times$  II<sup>-/-</sup> mice to levels comparable to those observed on DP thymocytes from control mice (Fig. 5 C).

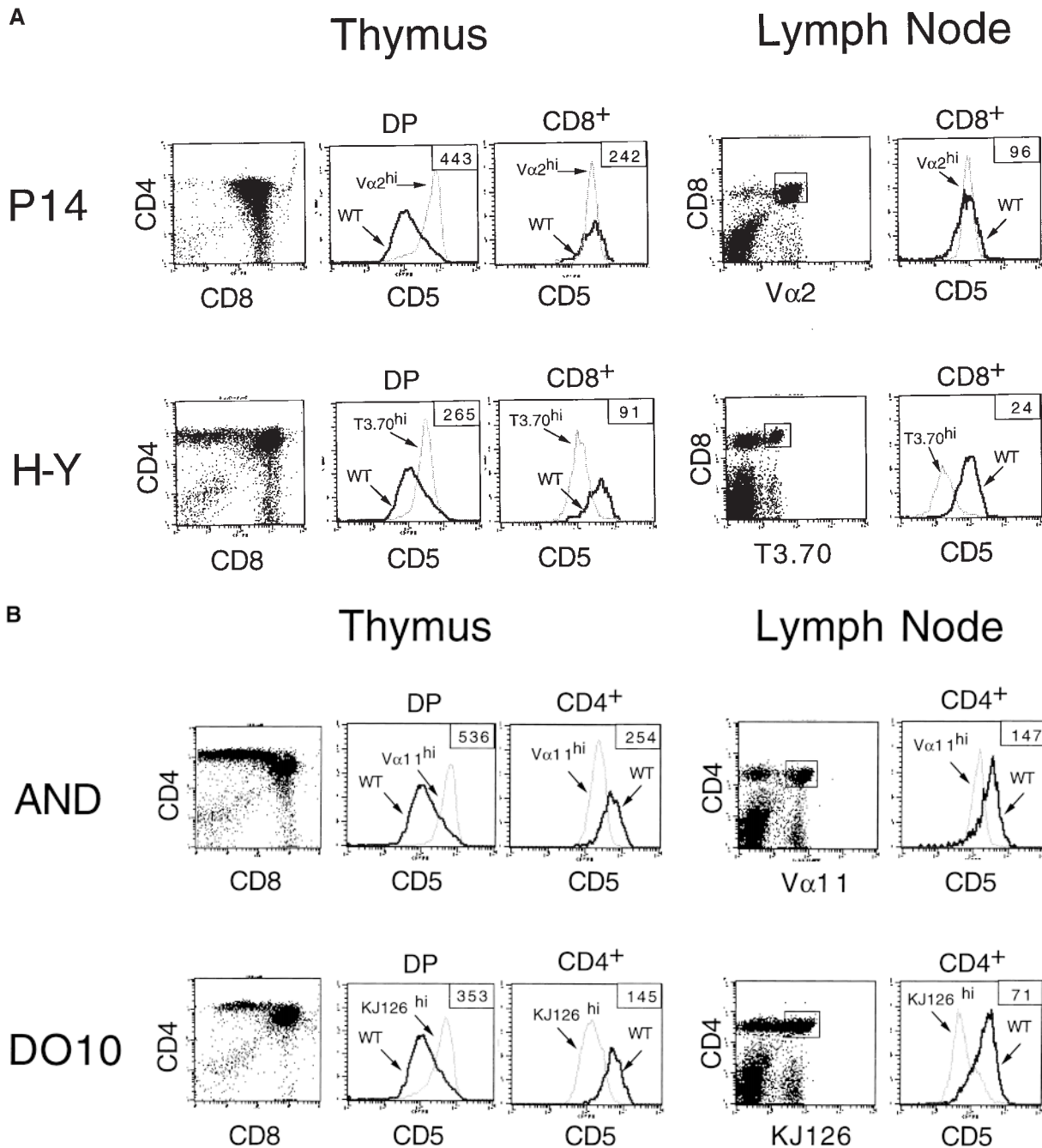
The relationship between TCR signal intensity and CD5 expression on DP thymocytes was examined by *in vitro* cross-linking with varying amounts of anti-CD3 $\epsilon$ . The results of this experiment revealed a direct relationship between the concentration of cross-linking antibody and CD5 surface levels (Fig. 4 B). The correlation between TCR signaling and CD5 induction could also be assessed using transgenic mice that express similar levels of TCR but whose TCRs differ in their signaling potential due to differences in the total number of ITAMs contained within the  $\zeta$  chain (30).  $\zeta$ <sup>-/-</sup> mice that have been reconstituted with a transgene encoding the full-length  $\zeta$  chain ( $\zeta$ -3 ITAM) express surface TCRs that contain a full complement of ITAMs (10 ITAMs per TCR complex), whereas reconstitution of  $\zeta$ <sup>-/-</sup> mice with a truncated  $\zeta$  chain transgene lacking all three ITAMs ( $\zeta$ -0 ITAM) results in surface expression of TCRs that contain only four ITAMs per TCR complex (provided by the CD3 chains) (30). Although the TCR levels on DP thymocytes from these transgenic mice are nearly identical, mean CD5 levels are significantly lower on the majority of DP thymocytes from  $\zeta$ -0 ITAM mice relative to the  $\zeta$ -3 ITAM mice (Fig. 4 B). Thus, the level of TCR surface expression and the intensity of the TCR signal quantitatively influence CD5 surface expression on DP thymocytes.

**CD5 Levels on SP Thymocytes and T Cells Are Dictated by the Avidity of the Positively Selecting TCR Interaction.** The observation that CD5 is upregulated to high levels on DP thymocytes *in vivo* after engagement of the TCR by ligands that promote either positive or negative selection, or *in vitro* by cross-linking of the TCR (references 32, 39–42; Fig. 4) suggested the possibility that CD5 surface expression might be quantitatively influenced by the affinity of the TCR for selecting ligand, together with the concentration of selecting ligand (TCR avidity). To address this question, we analyzed CD5 surface expression on thymocytes from two different MHC class I–restricted (H-Y, reference 26, and P14, reference 25) and two different MHC class II–restricted (DO10, reference 28, and AND, reference 27) TCR transgenic lines, each expressing distinct clonotypic TCRs. Under conditions known to promote positive selection, CD5 levels on DP thymocytes from all of the transgenic mice were higher than those expressed on the majority of DP thymocytes from nontrans-



**Figure 5.** CD5 surface expression on thymocytes from mice lacking MHC. (A) Histograms show CD5 and CD3 surface levels on gated CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocyte populations from mice lacking expression of MHC class I (*MHC I*<sup>-/-</sup>) or MHC class I and class II [*MHC (I × II)*<sup>-/-</sup>]. CD3 and CD5 levels on DP thymocytes from control B6 (WT) mice are included for comparison. (B) CD5 levels on CD4<sup>+</sup>CD8<sup>-</sup> (DN) and DP thymocytes from *MHC I*<sup>-/-</sup> and *MHC (I × II)*<sup>-/-</sup> mice. (C) Induction of CD5 by CD3 cross-linking. Thymocytes from C56 BL/6 (WT) and *MHC (I × II)*<sup>-/-</sup> mice were incubated in the presence (+2C11) or absence (-2C11) of plate-bound anti-CD3 $\epsilon$  antibody (50  $\mu$ g) for 18 h at 37°C and CD5 and CD69 expression on DP thymocytes was assessed by FACS<sup>®</sup>. Dotted lines represent staining with control antibodies.

genic mice (Fig. 6, A and B). As the shift in CD5 surface expression from CD5<sup>int</sup> to CD5<sup>hi</sup> on the bulk of DP thymocytes in transgenic mice is observed in either positively or negatively selecting but not in nonselecting backgrounds (data not shown), this result presumably reflects the fact that most DP thymocytes in TCR transgenic mice, but only a small percentage of DP thymocytes from non-TCR transgenic mice express TCRs that are engaged by selecting ligand (Fig. 6, A and B). However, gating on transgenic TCR<sup>hi</sup> SP thymocytes revealed clear differences in the level of CD5 expression among the various transgenic lines, with P14 > H-Y on CD8 SP thymocytes for the MHC class I–restricted TCRs, and AND > DO10 on the CD4 SP thymocytes for the MHC class II–restricted TCRs (Fig. 6, A and B). Significantly, the relative differences in CD5 surface expression were maintained on peripheral SP T cells generated by positive selection (CD8<sup>+</sup> T cells in the case of P14 and H-Y TCR transgenic mice, and CD4<sup>+</sup> T



**Figure 6.** CD5 expression on thymocytes and mature T cells from TCR transgenic mice. (A) Comparison of CD5 levels on thymocytes and lymph node T cells from MHC class I-restricted (P14 H-2<sup>b</sup> and H-Y H-2<sup>b</sup>) TCR transgenic mice. (B) Comparison of CD5 levels on thymocytes and lymph node T cells from MHC class II-restricted (AND H-2<sup>b\*<sub>k</sub></sup> and DO10 H-2<sup>d</sup>) TCR transgenic mice. Single-color histograms represent CD5 staining on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, as well as on CD8<sup>+</sup> SP thymocytes and T cells (HY and P14 TCR transgenic mice), or CD4<sup>+</sup> SP thymocytes and T cells (AND and DO10 TCR transgenic mice). Shown are CD5 levels on transgenic TCR<sup>hi</sup> gated cells. Data obtained for similar cell populations from non-transgenic (WT) mice are shown for comparison. Numbers in boxes depict mean CD5 fluorescence for the transgenic TCR<sup>hi</sup> cells.

cells in the case of AND and DO10 TCR transgenic mice; Fig. 6, A and B). Since TCR levels on SP T cells from the class I- or class II-restricted TCR transgenic mice were similar as assessed by staining with anti-CD3 $\epsilon$  or anti-TCR- $\beta$  antibodies (data not shown), these results suggested that the differences in CD5 levels might reflect dif-

ferences in the avidity of the positive selecting interaction (i.e., P14 > H-Y and AND > DO10). Although the natural positively selecting ligands for these TCRs are unknown, their relative avidity can be inferred by the efficiency of positive selection (43). Experimental data support the idea that within the “window” of TCR-ligand-MHC

interactions that promote positive selection, higher avidity interactions increase the efficiency of positive selection, resulting in the generation of increased numbers of TCR<sup>hi</sup> SP thymocytes (43). In the case of the H-Y and P14 transgenic TCRs, several observations indicate that the efficiency of positive selection is greater in P14 than in H-Y (female) TCR transgenic mice (25, 26). For example, both the total number of transgenic TCR<sup>hi</sup> thymocytes and the number of transgenic TCR<sup>hi</sup>CD8<sup>+</sup> thymocytes and T cells in P14 TCR transgenic mice exceeds that observed in H-Y transgenic female mice (Fig. 6 A). These observations suggest, although by no means prove, that positive selection is mediated by higher avidity TCR–ligand–MHC interactions in P14 transgenic mice than in H-Y (female) transgenic mice. Applying similar criteria, positive selection also appears to be mediated by a higher avidity TCR–ligand–MHC interaction in AND transgenic mice than in DO10 transgenic mice (Fig. 6 B).

To further investigate the possible relationship between CD5 expression and TCR avidity, we analyzed positive selection in the same transgenic line (DO10) under conditions that have been shown to alter the avidity of the positively selecting TCR–MHC–ligand interaction (44, 45). Thymocytes with DO10 TCR are positively selected in both the H-2<sup>d</sup> and H-2<sup>b</sup> backgrounds; however, the small thymus size and reduction in DP thymocytes observed in DO10, H-2<sup>b</sup> mice is consistent with the induction of partial clonal deletion as a result of increased TCR–MHC–ligand avidity interactions (44, 45). Significantly, the CD4<sup>+</sup> transgenic TCR<sup>hi</sup> (KJ-126<sup>hi</sup>) thymocytes and T cells generated in DO10 H-2<sup>bxd</sup> mice were found to express higher surface levels of CD5 than do CD4<sup>+</sup> KJ-126<sup>hi</sup> thymocytes and T cells from DO10 H-2<sup>dxid</sup> mice (Fig. 7 A). Notably, the KJ-126<sup>hi</sup>CD4<sup>+</sup> T cells from DO10 H-2<sup>bxd</sup> and DO10 H-2<sup>dxid</sup> express similar levels of  $\alpha/\beta$ -TCR and CD4 (Fig. 7 A). Thus, the difference in CD5 levels could not be attributed to differences in TCR or CD4 coreceptor surface expression. In addition, since the identical transgenic TCR was used in these experiments, the differences observed could not reflect variation in the timing or onset of transgene expression during thymocyte development. Rather, these results suggest that the level of CD5 expression on mature T cells is determined during development in the thymus by the avidity of the TCR for selecting ligand.

A prediction of this hypothesis is that the avidity of the positively selecting TCR–MHC–ligand interaction may regulate the level of CD5 surface expression by influencing the intensity of the TCR signaling response. To examine this relationship, we analyzed the effect on CD5 expression produced by lowering the TCR signaling potential from 10 to 4 ITAMs per TCR complex. For these experiments, we chose the P14 TCR transgene because prior experiments had revealed that significant numbers of P14 TCR<sup>hi</sup> (V $\alpha$ 2<sup>hi</sup>) CD8 SP T cells are generated in both  $\zeta$ -3 ITAM and  $\zeta$ -0 ITAM P14-TCR transgenic mice (Fig. 7 B). We found that lowering the TCR signaling capacity by removal of  $\zeta$  chain ITAMs ( $\zeta$ -0 ITAM tg versus  $\zeta$ -3 ITAM tg) resulted in the generation of V $\alpha$ 2<sup>hi</sup> CD8-SP cells that

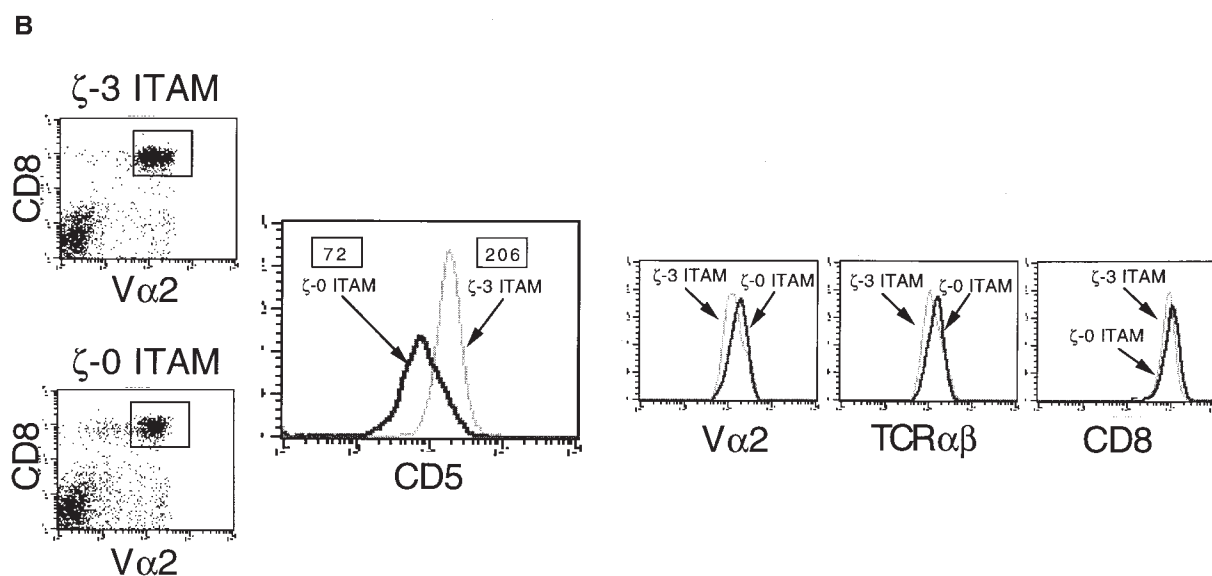
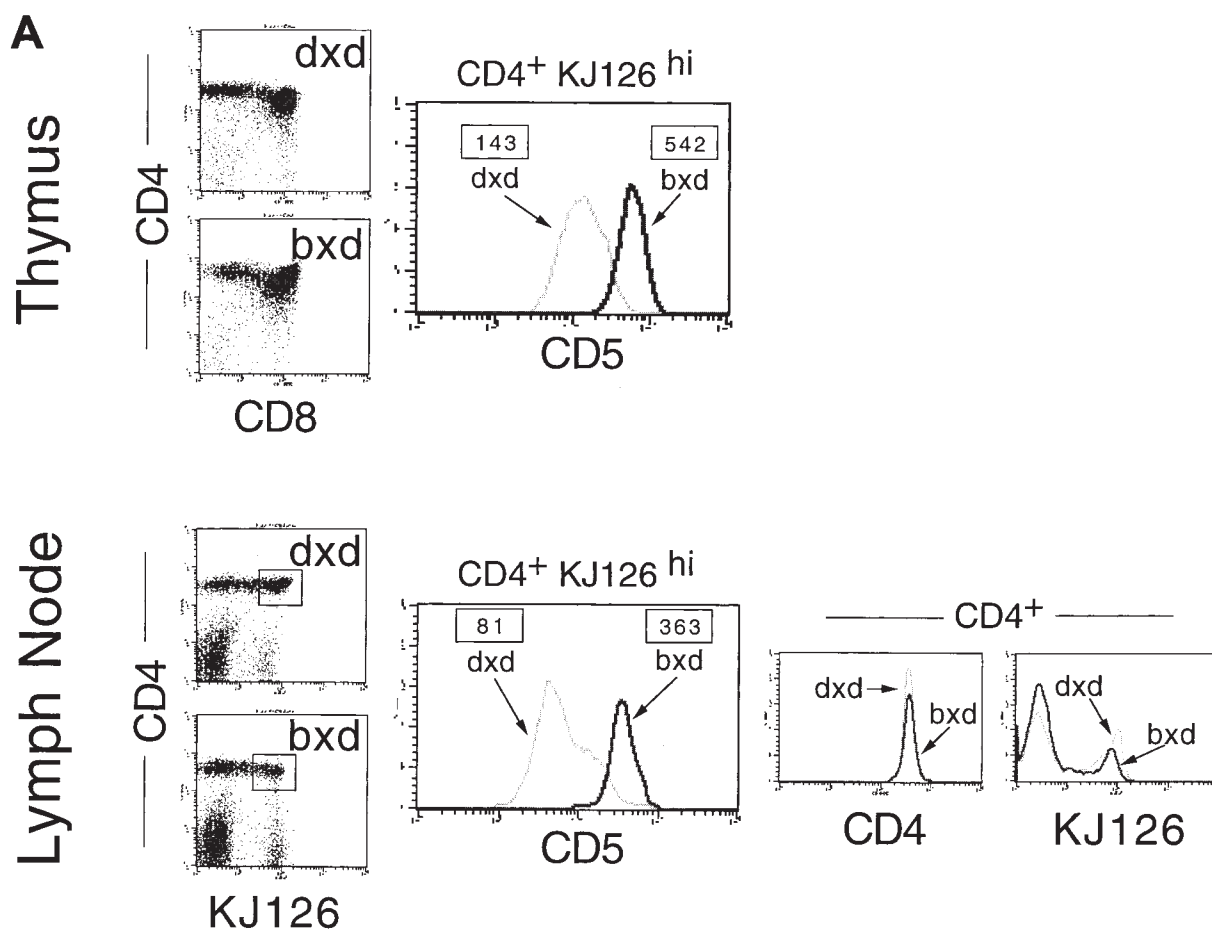
express lower levels of CD5 (Fig. 7 B). Taken together, these results indicate that CD5 surface expression is developmentally regulated in response to the avidity of the TCR for positively selecting ligands.

## Discussion

Interest in the role of CD5 in lymphocyte development has grown considerably in light of recent data suggesting that CD5 functions to negatively regulate signaling through the B and T cell antigen receptors (16, 17). In this study, we examined CD5 expression during normal thymocyte development and in mice with defects in thymocyte maturation. Our results demonstrate a critical role for pre-TCR and TCR signals, and notably TCR avidity, in regulating CD5 surface expression.

Early DN thymocytes express very low levels of CD5 independent of their ability to undergo TCR gene rearrangement, and therefore independent of their ability to express pre-TCR and/or TCR complexes. That the pre-TCR is not required for initial CD5 expression on early DN thymocytes is not surprising, as previous studies have documented very low levels of CD5 on pro-T cells in the thymus (46). Of significance, however, is that signals transduced by the pre-TCR upregulate CD5, and that the level of CD5 surface expression reflects the extent or intensity of pre-TCR signaling (Fig. 3 B; reference 47). Consistent with this observation, both p56<sup>lck</sup> and  $\zeta$  chain, which appear to act primarily by quantitatively influencing the pre-TCR signaling response (24, 29, 38, 47, 48), are required for full CD5 induction by the pre-TCR (29, 49; Fig. 3 C). Although these results suggest a possible role for CD5 at the DN–DP transition, perhaps through modulation of pre-TCR signaling threshold, the fact that thymocyte maturation appears unaffected in CD5<sup>-/-</sup> mice argues that this function is not critical for T cell development (50).

Interestingly, most DP thymocytes from TCR- $\alpha$ <sup>-/-</sup> mice (which can express pre-TCR complexes but are  $\alpha/\beta$ -TCR<sup>-</sup>) express nearly normal (intermediate) levels of CD5, whereas CD5 levels on DP thymocytes from MHC (class I  $\times$  II)<sup>-/-</sup> mice (which are  $\alpha/\beta$ -TCR<sup>int-hi</sup>) express low levels of CD5. We interpret these observations as indicating that continued signaling, either through the pre-TCR or  $\alpha/\beta$ -TCR, is required to maintain CD5 expression at intermediate levels on DP thymocytes. Thus in TCR- $\alpha$ <sup>-/-</sup> mice, continued expression of the pre-TCR provides this signaling function, whereas in MHC (class I  $\times$  II)<sup>-/-</sup> mice, replacement of the pre-TCR by TCR complexes that are incapable of generating signals by interacting with MHC leads to the downregulation of CD5. Results consistent with our own have also been reported by Dutz et al., who noted a specific requirement for MHC class I for the generation of CD5<sup>int</sup> DP thymocytes in class I–restricted H-Y TCR transgenic mice (51). The observation that most DP thymocytes from transgenic mice express intermediate levels of CD5 in a nonselecting background (51) further indicates that extremely low avidity or “non-cognate” TCR–MHC interactions are sufficient to main-



**Figure 7.** (A) Comparison of CD5 levels on CD4<sup>+</sup> thymocytes and T cells from DO10 TCR transgenic mice generated in the H-2<sup>dxd</sup> and H-2<sup>bxd</sup> backgrounds. *Top:* Two-color (CD4 versus CD8) analysis of thymocytes from DO10/H-2<sup>dxd</sup> and DO10/H-2<sup>bxd</sup> mice. Histogram depicts CD5 levels on CD4<sup>+</sup> KJ126<sup>hi</sup> thymocytes. *Bottom:* Two-color (CD4 versus KJ126) analysis of lymph node cells from DO10/H-2<sup>dxd</sup> and DO10/H-2<sup>bxd</sup> mice. Large histogram depicts CD5 levels on CD4<sup>+</sup> KJ126<sup>hi</sup> T cells. Small histograms show CD4 and KJ126-TCR levels on total CD4<sup>+</sup> T cells. Numbers in boxes depict mean CD5 fluorescence for the transgenic TCR<sup>hi</sup> cells. (B) Comparison of CD5 levels on CD8<sup>+</sup> T cells generated in P14 TCR transgenic mice expressing TCRs containing full-length ( $\zeta$ -3 ITAM) or signaling defective ( $\zeta$ -0 ITAM)  $\zeta$ -chains. Histograms depict V $\alpha$ 2, TCR- $\beta$ , CD8, or CD5 staining on gated CD8<sup>+</sup>V $\alpha$ 2<sup>+</sup> T cells (gates are shown in the two-color plots). Numbers in boxes depict mean CD5 fluorescence.



tain intermediate levels of CD5 expression on DP thymocytes.

CD5 is upregulated to high levels on a small subset of DP thymocytes, before TCR upregulation or CD4/CD8 lineage commitment as assessed by coreceptor downregulation (32, 39–41, 51). Data from our own and previous work indicate that this response is mediated by higher avidity TCR–MHC–ligand interactions than those that are required to maintain intermediate levels of CD5 expression on DP thymocytes (32, 39–41, 51). In agreement with this model, CD5<sup>hi</sup> DP thymocytes are absent both in TCR- $\alpha^{-/-}$  mice that lack  $\alpha/\beta$ -TCR and in MHC (class I  $\times$  II)<sup>-/-</sup> mice that express  $\alpha/\beta$ -TCR but are incapable of engaging ligand. Conversely, in TCR transgenic mice in which a defined TCR is expressed on a high percentage of DP thymocytes, a large cohort of CD5<sup>hi</sup> DP thymocytes is generated under conditions that promote either positive or negative selection but not in a nonselecting background (51). Thus, only those DP thymocytes that express TCRs that can interact with MHC–ligand with sufficiently high affinity to promote either positive or negative selection upregulate CD5 surface expression to high levels.

Although CD5 upregulation on DP thymocytes upon TCR engagement has been previously shown, the current study reveals that CD5 surface expression on SP thymocytes and T cells differs depending upon the clonotypic TCR that they express. Transgenic TCR<sup>hi</sup> SP T cells were found to express similar (P14), lower (H-Y, AND, D011.10/H-2<sup>d</sup>), or higher (D011.10/H-2<sup>bxd</sup>) levels of CD5 compared with mean CD5 expression on corresponding SP T cell populations from nontransgenic mice (Figs. 6 and 7). Our data also suggest that the level of CD5 expressed on SP thymocytes and T cells generated during positive selection corresponds to the avidity (signal intensity) of the positively selecting TCR–MHC–ligand interaction. When the avidity of the positively selecting TCR–MHC–ligand interaction was varied for the same TCR by changing the MHC haplotype or the signal intensity was varied by removal of TCR ITAMs, the results also supported this hypothesis. Because the antigen specificity of the transgenic TCRs used in this study should result in the generation of “naive” SP thymocytes and T cells that should not encounter their native antigen *in vivo*, our results also argue that CD5 levels are “fixed” during positive selection on the basis of TCR specificity and these relative levels are subsequently maintained on circulating mature SP T cells independent of engagement of the TCR by antigen. The mechanism by which CD5 expression levels are established during thymocyte development could involve modulation of CD5 in response to TCR signal intensity followed by

maintenance of this relative level of expression on mature T cells. Alternatively, it is possible that only those thymocytes that constitutively express the appropriate level of CD5 with respect to their clonotypic TCR are selected to mature to the SP stage. Since these mechanisms are not mutually exclusive, both may also be operative.

Since CD5 acts to attenuate signaling by the TCR (16), the capacity of cells to regulate CD5 surface levels could serve several important functions during development. For example, induction of CD5 on thymocytes and mature T cells after TCR engagement may serve to dampen the TCR signaling response, especially after restimulation. At the DP stage, upregulation of CD5 represents an early response to engagement of the TCR by selecting ligand as it precedes upregulation of the TCR (32, 40, 51). The rapid increase in CD5 levels after initial TCR engagement may act to inhibit subsequent TCR-mediated signaling responses. Increased CD5 expression in response to TCR engagement could also provide an off signal that participates in terminating the activation response. Another role suggested by our data is that CD5 levels may influence the triggering threshold for T cell activation. This function might be particularly important during thymocyte selection when the TCR signaling response dictates the fate of DP thymocytes (i.e., either positive or negative selection). Indeed, analysis of CD5-deficient mice has shown that CD5 does participate in thymocyte selection through its ability to negatively regulate the TCR signaling response (16). Interestingly, the effect of CD5 deletion on thymocyte selection varied among different TCR transgenic mice, resulting in enhanced positive selection in H-Y TCR transgenic mice and a shift from positive selection to partial negative selection in P14 TCR transgenic mice (16).

The ability of thymocytes to regulate CD5 surface expression also suggests that the relative impact of CD5 on TCR signal transduction can be varied in relation to the avidity of the TCR for selecting ligand, providing a mechanism for fine tuning of the TCR signaling response. Surface levels of both CD4 and CD8, which unlike CD5 provide costimulatory signals, also have been shown to vary among individual SP T cells that express distinct TCRs (52). An advantage of using multiple surface structures such as CD4, CD8, and CD5 to influence the overall signaling response delivered to thymocytes and T cells could be to enable a much broader range of TCRs to transduce signals appropriate for positive selection than would otherwise be possible if the affinity of the TCR for selecting ligand alone dictated the outcome. Thus, the potential for TCR signal modulation by CD5 may be particularly useful for generating the maximum possible diversity in the mature T cell repertoire.

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